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A Direct and Practical Approach to Assessing the Impact of Emulsion Composition on Vitamin A Stability

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ABSTRACT

Vitamin A is an essential micronutrient involved in vision, immunity, and growth. Despite its widespread use in food, cosmetic, and pharmaceutical products, vitamin A is highly prone to oxidation due to its conjugated double bonds, leading to reduced biological activity and efficacy. While various formulation strategies have been explored to enhance its stability, there is a notable lack of stability data and understanding of vitamin A oxidation, particularly in dispersed systems. This study aimed to evaluate the oxidative stability of vitamin A in model emulsions and identify how emulsion composition affects its degradation. Studying the influence of emulsion composition provides a better understanding of the possible oxidation pathways, including a nonradical pathway. An innovative method combining gentle emulsification via solvent displacement with real-time degradation monitoring was used. Retinyl palmitate (RP) demonstrated the highest stability compared to retinol (RO) and retinyl acetate (RA), due to structural and electronic factors. Among emulsifiers, the cationic type slightly improved stability by repelling positively charged pro-oxidant molecules. Three phenolic antioxidants, α-tocopherol (TOH), butylated hydroxytoluene (BHT), and carnosic acid (CA), improved stability, with TOH being the most effective. However, early-stage degradation could not be completely prevented, suggesting the existence of a predominant nonradical degradation pathway. The impact of iron (Fe^{2+}) was minimal and attributed to the low hydroperoxide production, reinforcing the hypothesis of a nonradical initiation. Additionally, electrostatic repulsion in positively charged emulsions further limited iron's pro-oxidant effect. These findings enhance our understanding of vitamin A oxidation mechanisms and highlight potential stabilization strategies for its formulation in emulsified systems.

1 | Introduction

Vitamin A is an essential micronutrient found in the diet, either from animal sources (fish liver oils, dairy products, eggs, and meat) or plant sources (carrot, pumpkin, spinach, mango) containing provitamin A carotenoids (Carazo et al. 2021; D'Ambrosio et al. 2011). In humans, it contributes to numerous biological functions such as visual acuity, immune system maintenance, fetal development, and growth of young children (Blomhoff and Blomhoff 2006). The nutritional reference values for the population are 750 μg per day for men, 650 μg for women, and 450–550 μg for children (ANSES 2012).

Abbreviations: Aox, antioxidant; BHT, butylated hydroxytoluene; Brij35, polyoxyethylene(23)lauryl ether; CA, carnosic acid; DLS, dynamic light scattering; HLB, hydrophilic–lipophilic balance; noAox, no antioxidant; PDI, polydispersity index; RA, retinyl acetate; RO, retinol; RP, retinyl palmitate; SD, standard deviation; SDS, sodium dodecyl sulfate; Tdia, tallow diamine; TOH, α-tocopherol; VA, vitamin A.

Populations in impoverished countries, severely affected by malnutrition, are at the highest risk for vitamin A deficiency. It is estimated that 250 million children suffer from this deficiency (WHO 2024).

Vitamin A refers to a group of fat-soluble compounds also known as retinoids, characterized by an unsaturated isoprenoid chain containing five conjugated double bonds. The biologically active forms of vitamin A include retinol (RO), retinal, and retinoic acid (Carazo et al. 2021) (Figure 1a–c), the all-trans isomers having the highest biological activities and representing two-thirds of natural vitamin A sources. Additionally, vitamin A can occur in esterified forms, such as retinyl acetate (RA) and retinyl palmitate (RP), also called vitamin A acetate (Figure 1d) and vitamin A palmitate (Figure 1e), respectively. Finally, carotenoids are not strictly vitamin A, but provitamin A, meaning they serve as precursors to the active forms of vitamin A. Due to the electron-rich conjugated double bonds in its carbon chain, vitamin A is highly susceptible to oxidative degradation reactions. Indeed, exposure to oxygen, light, heat, prooxidants, and the presence of water and acidic compounds can accelerate its degradation, resulting in the loss of its biological properties and efficacy (Loveday and Singh 2008).

In view of its numerous health benefits, vitamin A supplementation is widely used in manufactured food products such as infant milk formulas (Cancalon et al. 2024) or in animal feed supplementation (Galli et al. 2024) to enhance the nutritional profile.

In recent years, Vitamin A has also been extensively implemented in cosmetics and skincare products due to its numerous dermatological benefits. Indeed, it was demonstrated to be an effective antiaging molecule (Zasada and Budzisz 2019) and a potent photoprotective agent against deleterious UV rays (Antille et al. 2003). Furthermore, vitamin A is also used in clinical treatments for psoriasis, hyperkeratosis, and acne (European Commission. Directorate General for Health and Food Safety 2021).

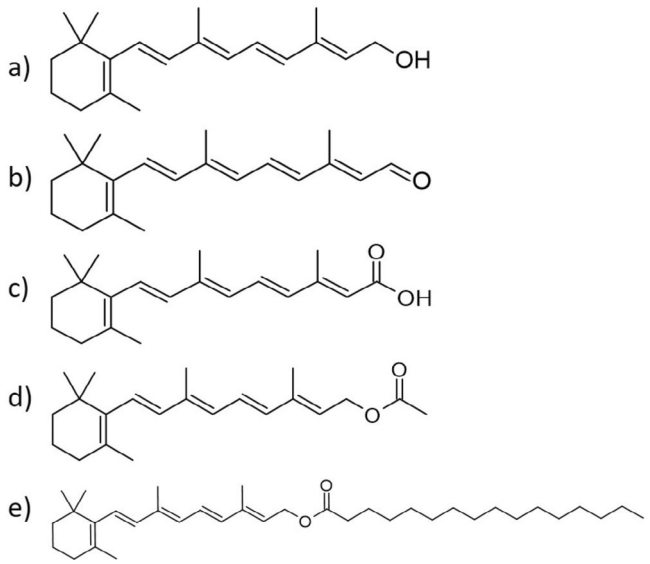

FIGURE 1 | Chemical structure of (a) Retinol, (b) Retinal, (c) Retinoic acid, (d) Retinyl acetate, and (e) Retinyl palmitate.

However, all of these products have faced significant challenges regarding the oxidative stability of vitamin A. Various strategies are generally implemented to prevent premature oxidation, including the use of antioxidants (AOx) such as butylated hydroxytoluene (BHT), α-tocopherol (TOH), thylenediaminetetraacetic acid (EDTA), and sodium ascorbate (Banasaz et al. 2021; Yoshida et al. 1999). In addition, the improvement of vitamin A oxidative stability has also been considered through encapsulation strategies in various systems such as carbohydrate core-shell (Fallahasghari et al. 2023), double layer microcapsules (Albertini et al. 2010), or solid lipid nanoparticles (Jung et al. 2013), to name a few.

Despite these strategies, the Scientific Committee on Consumer Safety of the EU considers that there is a lack of stability data and knowledge on vitamin A oxidation (European Commission. Directorate General for Health and Food Safety 2021). This knowledge gap has also been pointed out by Xu and Watson (2021), who studied microencapsulated vitamin A palmitate and asserted that progress in research on retinyl ester stabilization and degradation pathways is still insufficient, and also by Temova Rakuša et al. (2021), who revealed retinoid instabilities in commercial cosmetic creams and serums. In most commercial products, vitamin A is present in a dispersed system or passes through a dispersed phase before being dried. However, the mechanisms of oxidation, particularly in dispersed media, remain insufficiently explored, as most of the current studies focus on bulk oil or pure forms. In dispersed systems, the exchange area of the dispersed phase is significantly increased, making it more vulnerable to degradation. Moreover, in aqueous environments, vitamin A is highly prone to oxidation, as the diffusion of pro-oxidant species is enhanced, increasing its exposure to oxidative degradation. Given this observation, enhancing our understanding of vitamin A oxidation in dispersed media is crucial for developing effective stabilization strategies in food, cosmetic, and pharmaceutical formulations. The present study was designed as a direct and practical tool for evaluating the oxidative stability of vitamin A in model emulsions by identifying how emulsion composition influences its stability. To achieve this, we employed an innovative method with a gentle emulsification process by solvent displacement, combined with real-time degradation monitoring. Moreover, we investigated compositional factors previously identified as influencing carotenoid stability (Boon et al. 2010) given that the structure of vitamin A is similar to that of carotenoids and that, as a result, the oxidation mechanisms are assumed to be similar. This approach allowed us to investigate the influence of four key compositional factors: (i) the chemical form of vitamin A, (ii) the type of emulsifier, (iii) the role of AOx, and (iv) the presence of iron.

In the first part of the study, RO, RA, and RP stability was evaluated as these three forms are the more commonly found in vitamin A-based products. Subsequently, the influence of different emulsifiers on vitamin A acetate stability was assessed, given the type of emulsifiers was proved to impact carotenoids stability (Boon 2014). Finally, the protective effects of three phenolic AOx were investigated: two widely used synthetic AOx, namely butylhydroxytoluene (BHT) and TOH, were chosen due to their large use in the food industry, and carnosic acid (CA), a naturally sourced antioxidant derived from rosemary, which has gained increasing interest. Finally,

TABLE 1 | Compositions of emulsions.

	Vitamin A			Emulsifiers			Antioxidants		
CODE	Retinyl acetate (μM)	Retinol (μM)	Retinyl palmitate (μM)	Tallow diamine[a] (μM)	SDS (μM)	Brij35[b] (μM)	BHT (μM)	TOH (μM)	CA (μM)
E_RA	60	—	—	6.4	—	—	—	—	—
E_RO	—	60	—		—	—	—	—	—
E_RP	—	—	60		—	—	—	—	—
E_noSF	60	—	—	—	—	—	—	—	—
E_Tdia		—	—	6.4	—	—	—	—	—
E_SDS		—	—	—	6.4	—	—	—	—
E_Brij35		—	—	—	—	6.4	—	—	—
E_noAox		—	—	6.4	—	—	—	—	—
E_BHT1		—	—		—	—	1	—	—
E_BHT2.5		—	—		—	—	2.5	—	—
E_BHTH5		—	—		—	—	5	—	—
E_TOH0.05		—	—		—	—	—	0.05	—
E_TOH0.25		—	—		—	—	—	0.25	—
E_TOH0.5		—	—		—	—	—	0.5	—
E_TOH1		—	—		—	—	—	1	—
E_TOH2.5		—	—		—	—	—	2.5	—
E_TOH5		—	—		—	—	—	5	—
E_CA0.25		—	—		—	—	—	—	0.25
E_CA0.5		—	—		—	—	—	—	0.5
E_CA1		—	—		—	—	—	—	1
E_CA5		—	—		—	—	—	—	5

[a]Average molar mass: 312.58 g.mol^{-1}.
[b]Average molar mass: 1199.54 g.mol^{-1}.

for all emulsions, the impact of the presence of ferrous ions in the continuous phase was systematically examined, as iron is a well-known pro-oxidant that catalyzes hydroxyl radical formation during lipid peroxidation (Minotti and Aust 1987) and has been implicated in carotenoid oxidation pathways (Boon et al. 2010).

2 | Materials and Methods

2.1 | Chemicals

RO 99.1%, RA 99.9%, RP 97%, BHT 99%, TOH 98%, CA 99.9%, sodium dodecyl sulfate (SDS) 99%, Polyoxyethylene (23) dodecyl ether (Brij35), anhydrous iron chloride (II) 98%, 1,4-dioxane (dioxane) ≥ 99%, and propan-2-ol (isopropanol) HPLC-grade were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). HPLC-grade water was purchased from Carlo Erba (Val de Reuil, France). A tallow diamine (Tdia) (CAS: 61791–55-7) was supplied from Arkema (La Garenne-Colombes, France).

2.2 | Emulsion Preparation

2.2.1 | Organic Phase

The organic phases were prepared in two steps. First, all compounds were dissolved individually in dioxane (or dioxane/water 9:1 v/v for emulsifiers) in order to obtain the following stock solutions:

- Vitamin A (each form): 87 mM
- Emulsifiers (Tdia, SDS, Brij35): 80 mM
- AOx (TOH, BHT, CA): 0.9–9.0 mM

Then, the solutions were mixed and adjusted to 3.5 mL with dioxane, in such proportions that the composition of the final emulsion matches the values given in Table 1. The 60 μM vitamin A concentration allowed UV–vis absorbance to remain within the optimal detection range while still enabling subsequent extraction for further analysis. The 6.4 μM molar concentration of

emulsifier corresponds to 10% by mass of Tdia relative to vitamin A. The concentrations of the other emulsifiers were set to the same molar amount, which reflects typical levels commonly used to stabilize emulsions. Antioxidant concentrations ranged from 0.05 to 5 μM and were adjusted based on their relative efficacy. These concentrations reflect typical levels used to protect substrates against oxidation.

2.2.2 | Emulsification Process

A total of 150 mL of HPLC grade water was stirred in a 250 mL beaker using an L5M Silverson homogenizer (Silverson Machines Ltd., UK) with a 1″ L4/L5 tubular mixing unit with disintegration head and high-shear square-hole grid at a speed of 8000 rpm and ambient temperature for 1 min. Then, 600 μL of the corresponding organic phase was manually injected into the aqueous phase with a micropipette while maintaining stirring at the same speed. The homogenization process was continued for an additional 2 min after organic phase injection. The resulting emulsions have a final vitamin A concentration of 60 μM. The concentrations of each compound in the emulsions are given in Table 1.

2.3 | Monitoring of Vitamin A Degradation

A volume of 250 μL of emulsion was dispensed into 96 wells UV-star plastic transparent microplate (Greiner bio-one, Germany). Subsequently, 20 μL of either deionized water or 1.715 mg/L^{-1} ferrous chloride solution were added to each well, resulting in a final ferrous iron concentration of 0 or 1 μM. Vitamin A degradation kinetics were monitored by absorbance reading at 330 nm in a microplate reader (Spark TECAN, Switzerland) with reading taken every 10 min over a 10-h period. The temperature inside the microplate reader was maintained at 37°C throughout the experiment (This temperature was selected to be closed to physiological conditions and to have some satisfactory kinetics in terms of vitamin A degradation.). An orbital shaking step of 10 s was performed prior to each absorbance measurement to ensure consistent mixing of the solutions.

Result expression:

To normalize the results, raw absorbance was converted into relative absorbance:

$$\text{Relative absorbance } Ar\,(\%) = \frac{A_t - A_{\text{water}}}{A_0 - A_{\text{water}}} \times 100$$

where A_t and A_0 are the absorbances measured at time t and 0 min, respectively, and A_{water} corresponds to the absorbance of HPLC grade water.

The kinetics are presented by the percentage of remaining vitamin A, as a function of time:

$$\text{Vitamin A retention } (\%) = \frac{Ar_t}{Ar_{t0}} \times 100$$

where Ar_t and Ar_0 are the relative absorbances measured at time t and 0 min, respectively.

2.4 | Emulsion Characterization

2.4.1 | Droplet Size Measurement

The droplet size distribution of emulsions was measured by Dynamic Light Scattering (DLS) using Zetasizer pro (Malvern Panalytical, France). A 2 mL aliquot of emulsion was placed into a polystyrene cuvette (Fisherbrand, Fisher Scientific, France). A 3-min stabilization period was allowed prior to measurement. The refractive index was set to 1.55 for the material and 1.33 for the dispersant (water). The general-purpose analysis model was applied for the measurements performed at 25°C in triplicate, and results were reported as Z-average size (nm) and polydispersity index (PDI).

2.4.2 | Zeta Potential Measurement

Zeta potential was measured with a Zetasizer pro (Malvern Panalytical, France) in Zetasizer nano series disposable folded capillary cells DTS1070 (Malvern, UK). Cells were prior flushed three times with deionized water and isopropanol. Once isopropanol was fully evaporated, the cell was filled with the sample using syringes. Measurements were carried out at 25°C in triplicate with auto-mode analysis model. Minimum run was set to 10 and maximum run was set to 30.

2.4.3 | Physical Stability Evaluation of the Emulsion

To evaluate the physical stability of the emulsions with different emulsifiers, during the whole oxidation kinetics measurement, 10 mL of each emulsion were poured into a 30 mL brown flask and incubated for 17 h at 37°C in a shaking incubator (IKA, Germany) with orbital shaking (40 rpm). After 17 h (corresponding to the maximum time of analysis), DLS measurement was performed to check the physical stability of the emulsions (droplet sizes).

2.5 | Statical Analyses

Each emulsion was formulated in triplicate, and the measurements were performed three times on each of the triplicates. The results are presented as mean ± SD. Statistical significance was determined by one-way ANOVA and Student t test using XLSTAT and GraphPad prism. Values with different superscript letters (a, b, c, ...) are significantly different ($p < 0.05$).

3 | Results and Discussion

This study is designed as a direct and practical tool to evaluate the oxidative stability of vitamin A in emulsified systems and to identify how emulsion composition affects its stability. Conventional emulsification processes typically involve dispersing preheated vitamin A in an oil matrix before emulsification in an aqueous phase. However, this approach presents several drawbacks, including high material consumption, the risk of premature vitamin A degradation due to heating, and potential interactions between the oil matrix and vitamin A that may

accelerate its degradation. To overcome these limitations, we employed an innovative method with a gentle emulsification process by solvent displacement, coupled with real-time degradation monitoring, to ensure a more controlled and efficient evaluation of vitamin A stability.

The emulsification by solvent displacement method (also known as nanoprecipitation) is a technique used to form emulsions, particularly nano- and micro-emulsions, without the need for high-energy input such as heating or extensive mechanical shear as high-pressure homogenization. It involves the use of water-miscible organic solvents and its instantaneous diffusion into an aqueous phase leads to droplet formation (Mora-Huertas et al. 2011). Due to its density and boiling temperature approximating that of water, dioxane was selected as the organic solvent. It allows for the reduction of sampling volume variability compared with solvents of higher or lower density; it is water-soluble and facilitates the solubilization of all substrates in the study. In our model emulsions, the final volume of dioxane represents only 0.4% v/v, which can be considered negligible and not expected to affect the emulsion. Therefore, residual dioxane (which has been documented toxicological effect) does not need to be removed, as the emulsions are designed as research tools to identify how emulsion composition affects vitamin A stability rather than for direct food applications. This process facilitates the preparation of low-concentration vitamin A emulsions, thereby enabling real-time oxidation monitoring of multiple emulsions concurrently thanks to the use of a microplate reader in the UV domain. In addition, it serves to mitigate the risk of premature degradation due to prolonged extraction times, exposure to heat and light, and thus bias in oxidative stability results.

This novel, direct, and easy-to-use approach allowed us to study the impact of four main compositional factors on vitamin A stability: (i) the chemical form of vitamin A, (ii) the type of emulsifier, (iii) the role of AOx, and (iv) the presence of iron. The average droplet size and PDI of all emulsions were measured and are given in Table 2. The addition of emulsifiers and AOx did not impact the pH of the emulsions, which remained neutral in all emulsions.

TABLE 2 | Droplet size by z-average and polydispersity index (PDI) of emulsions.

CODE	Z-average (nm) ± SD	PDI	
VA chemical forms			
E_RA	$150^{a} \pm 9$	0.06	
E_RO	$178^{bc} \pm 2$	0.19	
E_RP	$194^{c} \pm 9$	0.29	
Emulsifiers			
E_noSF	$151^{a} \pm 7$	0.08	
E_Tdia	$139^{a} \pm 7$	0.07	
E_SDS	$137^{a} \pm 7$	0.06	
E_BRIJ35	$135^{a} \pm 8$	0.11	
Antioxidants			Statistical group*
E_noAox	$152^{abc} \pm 10$	0.06	α
E_BHT1	$164^{a} \pm 14$	0.04	α
E_BHT2.5	$153^{abc} \pm 9$	0.04	
E_BHTH5	$173^{a} \pm 3$	0.05	
E_TOH0.05	$171^{a} \pm 3$	0.06	α
E_TOH0.25	$160^{a} \pm 4$	0.06	
E_TOH0.5	$163^{a} \pm 3$	0.08	
E_TOH1	$157^{ab} \pm 12$	0.06	
E_TOH2.5	$155^{ab} \pm 7$	0.06	
E_TOH5	$168^{a} \pm 7$	0.08	
E_CA0.25	$133^{bcd} \pm 8$	0.05	β
E_CA0.5	$129^{cd} \pm 9$	0.06	
E_CA1	$120^{d} \pm 2$	0.08	
E_CA5	$133^{bcd} \pm 4$	0.08	

Note: Statistical significance was determined by one-way ANOVA and Student *t* test using XLSTAT and GraphPad prism. Values with different superscript letters (a, b, c...) are significantly different ($p < 0.05$).
*Statistical group on z-average means by antioxidant.

3.1 | Stability of VA Chemical Forms

Retinol, one of the active forms of vitamin A, is naturally present in the diet and constitutes the predominant form of vitamin A in the human body. However, RA, one of its esterified forms, is more widely used in food supplementation due to its stability and ease of formulation. RP, another esterified form, is also extensively employed in cosmetics and pharmaceuticals, and to a lesser extent in food supplementation. Upon ingestion, both RA and RP undergo enzymatic hydrolysis to yield RO, which can then be metabolized into other biologically active forms of vitamin A.

Ihara et al. (1999) studied the stability of RO and RP in ethanolic solution under both air oxidation and photolysis using fluorescent light. They revealed that RP was physicochemically more labile to photolysis but more resistant to air oxidation than RO. Furthermore, vitamin A loss kinetics were found to be dependent on the chemical forms of retinoids present in infant follow-on formulas model, with RO being the more susceptible to oxidation (Cancalon et al. 2024). However, no significant difference was observed between the stability of RO- and RP-based cosmetic creams after 6 months of storage at 25°C, suggesting that the most impactful factor is not the nature of vitamin A but the way it is formulated (Temova Rakuša et al. 2021).

In our study, the oxidative stability of RO, RA, and RP was compared in emulsions made by the solvent displacement method. The solvent displacement method was first described by Fessi et al. (1989). It generally requires only low-energy homogenization and is considered convenient, fast, and economically efficient for producing stable nanoparticles (Ganachaud and Katz 2005; Benbakrim et al. 2025; Mora-Huertas et al. 2011) The emulsions contained 60 μM of the corresponding form of vitamin A and 6.4 μM of Tdia as emulsifier (Table 1). This

bio-sourced compound is a versatile emulsifier used in a wide range of applications, including cosmetics and animal feed supplements. Its high HLB of 15.6, combined with cationic charge and alkaline surface environment, was selected to ensure good stability of emulsions when comparing vitamin A forms.

The z-average of emulsions containing RA (E_RA), RO (E_RO) and RP (E_RP) was $150^{a} \pm 11$ nm, $178^{bc} \pm 2$ nm, and $194^{c} \pm 11$ nm, respectively (Table 2). The three emulsions exhibited a narrow-centered monomodal intensity distribution with PDI proportional to droplet size, with E_RA having the lowest droplet size, followed by E_RO and E_RP, which is not significantly different from E_RO.

The larger droplet size observed could be associated with molecular size as E_RP has both the highest droplet size and molecular weight. These results indicate that, in the presence of Tdia as emulsifier, vitamin A self-assembles into droplets, forming a microemulsion. The ability of vitamin A to self-organize in water has also been reported in the literature (Bryl et al. 1998; Drabent et al. 1997), where RP introduced into water via 1,4-dioxane formed a stable dispersion with an effective vitamin A concentration 16 times higher than the solubility-limited value.

Since it has been demonstrated that the emulsions exhibit similar physicochemical properties, it was therefore possible to compare the chemical stability of the three forms of vitamin A under the same condition, a 10-h oxidation kinetics at 40°C in the dark (Figure 2). Given that iron is a well-established pro-oxidant, known for catalyzing hydroxyl and peroxyl radicals formation in lipid peroxidation (Minotti and Aust 1987) and playing a role in carotenoid oxidation pathways (Boon et al. 2010), its effect on vitamin A was also investigated with ferrous ions at 1 μM in the aqueous phase.

Oxidative kinetics depending on vitamin A forms (Figure 2) showed that with a retention of $38^{a} \pm 1\%$ after 10 h in the iron-free emulsion, RO (E_RO) appeared to be the least stable form of vitamin A, followed by RA (E_RA, $48^{b} \pm 2\%$ retention). In contrast, RP (E_RP) exhibited a significantly greater stability with a retention of $93^{c} \pm 2\%$ under the same conditions.

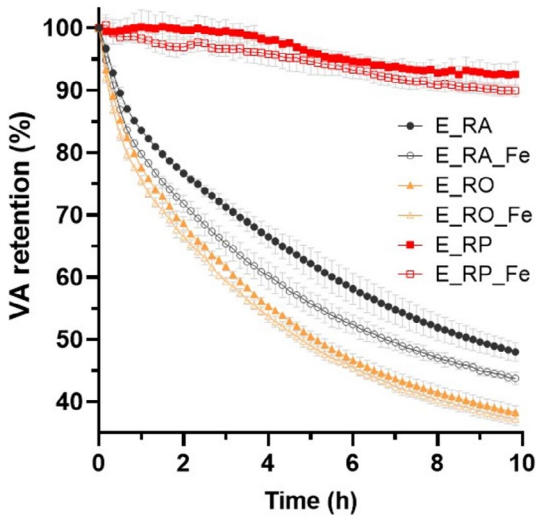

FIGURE 2 | Vitamin A degradation kinetics as a function of vitamin A form in the absence or the presence of iron (_Fe) (for used abbreviations see Table 1).

These findings are consistent with the study of Ihara et al. (1999), which reported that in ethanol solution under air oxidation, RP was more resistant than RO. They also align with the results from Cancalon et al. (2024), who found that among RA, RP, and RO, the latter was the least stable.

The difference in stability between vitamin A forms can be explained by several structural and electronic factors. First, the substitution of a hydroxyl (-OH) group by an ester (-O-COR) reduces chemical reactivity, making RA more stable than RO (Vojtko and Tomčík 2014). Additionally, the longer palmitate chain in RP provides further stabilization. This may be due to its inductive effect, which alters electron density around the carbonyl group, reducing its susceptibility to oxidation. The palmitate chain may also offer steric protection, shielding the conjugated double bonds from free radical attacks, thereby slowing oxidation. Furthermore, its bulky structure could limit interactions with pro-oxidant metals, reducing oxidative degradation.

In the presence of iron (Figure 2), the results were quite similar to those observed in the absence of iron for both RO (E_RO_Fe), with a retention of $37^{a} \pm 1\%$, and RP (E_RP_Fe) with a retention of $90^{c} \pm 1\%$. A slightly lower retention of $44^{d} \pm 1\%$ was observed for RA (E_RA_Fe) compared to its iron-free counterpart ($48^{b} \pm 2\%$). These findings regarding the limited impact of iron contrast with some previous literature. Indeed, some findings suggest that iron may directly interact with carotenoids, leading to the formation of degradation products (Boon et al. 2010). Additionally, the oxidative role of ferrous ions is well documented and primarily attributed to their ability to generate hydroxyl radicals (•OH) through two major pathways:

1. The Haber–Weiss reaction, where Fe^{2+} reacts with molecular oxygen (O_2) to produce hydroxyl radicals. However, in an emulsion system, these radicals are generated in the continuous aqueous phase, making it unlikely that they diffuse effectively to the interface where vitamin A oxidation primarily occurs.
2. The Fenton reaction, where Fe^{2+} catalyzes the decomposition of lipid hydroperoxides into highly reactive radicals, produces hydroxyl radicals at the oil – water interface. In this case, hydroxyl radical production requires the presence of lipid hydroperoxides, primarily oxidation products formed through radical pathways, within the dispersed phase or in the emulsifier layer.

This suggests that for RO, the presence of iron has virtually no effect, likely because its autoxidation (initiation) is inherently faster and/or significantly exceeds the rate of hydroperoxide formation and accumulation. The rate of hydroperoxide formation during RA oxidation might be higher than that of RO, which could explain why it is more sensitive to the presence of iron. Meanwhile, as RP is highly resistant to oxidation, its degradation remains minimal, preventing the formation of hydroperoxides, which in turn limits the pro-oxidant action of iron. This suggestion aligns with previous literature where oxidative products of vitamin A were mainly identified as aldehyde, epoxide,

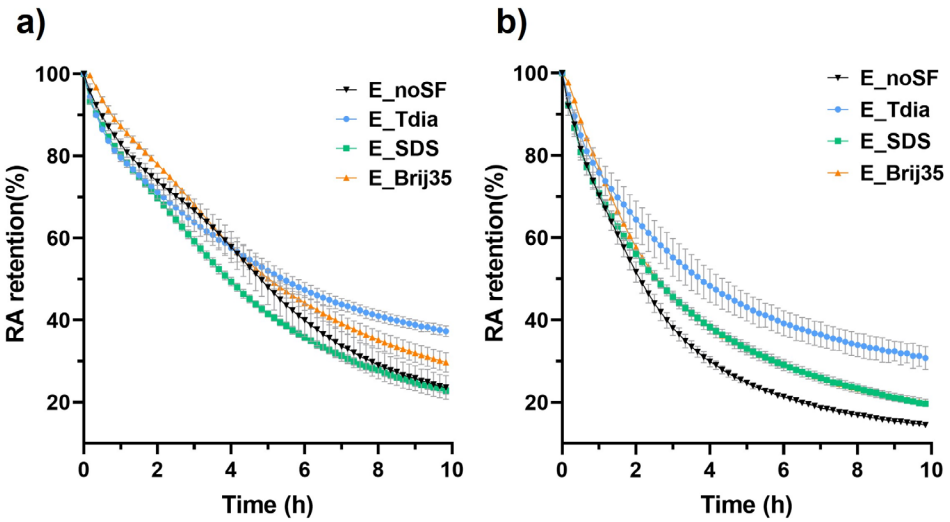

FIGURE 3 | Degradation kinetics of retinyl acetate depending on emulsifier type, (a) in absence of iron, (b) in the presence of iron (for used abbreviations see Table 1).

and vitamin A dimers (Crank and Pardijanto 1995; Crouch et al. 1992; Xu and Watson 2021) and not as hydroperoxides.

However, in our model emulsions at neutral pH, the tallow-diamine-based emulsifier surrounding the vitamin A droplets is positively charged, as are ferrous ions. It is therefore likely that electrostatic repulsion at the oil–water interface prevents iron from interacting with vitamin A, thereby limiting its pro-oxidant effect.

To sum up, the three tested forms of vitamin A successfully formed homogenous dispersion in water. However, emulsions containing RO and RA exhibited high susceptibility to oxidation, whereas RP demonstrated excellent stability. These results allow us to highlight two hypotheses: interface charge repulsion minimizes iron's oxidative and/or low hydroperoxide concentration at the interface further contributes to the lack of iron-induced degradation. Given its widespread use in food, cosmetic, and pharmaceutical industries, RA was selected for further investigation. To assess its stability under different conditions, formulations incorporating various emulsifiers and AOx were tested, providing insights into how these components influence RA degradation.

3.2 | Effect of Emulsifier

In this study, the impact of the presence and the nature of emulsifier on RA stability was investigated. Three commonly used emulsifiers were selected according to their ionic state. A Tdia, SDS, and polyoxyethylene(23)lauryl ether (Brij35) were used as emulsifiers. At neutral pH, the Tdia is in its protonated form and positively charged, as the pKa of primary and secondary amine groups is above 10. SDS is negatively charged with SO_4^- function, and Brij35 has a nonionic ethylene oxide head. All emulsifier concentrations in emulsions were set at 6.4 μM, far below their critical micellar concentration (8.2 mM for SDS, 0.209 mM for Brij35 and, for Tdia, estimated to 0.3 mM from octadecylamine hydrochloride, (Mukerjee 1971)) to limit micelle formation playing a significant role in the lipid oxidation pathway (Villeneuve et al. 2023). The impact of the nature and the charge of emulsifier on RA stability was investigated in the presence and absence of iron. An emulsion with no emulsifier (noSF) was used as a control. Emulsions were characterized by droplet size and PDI (Table 2). Additionally, interface charge (zeta potential) was measured.

The droplet size of emulsion without an emulsifier (E_noSF) was $151^a \pm 7$ nm, while it was $139^a \pm 7$ nm for the emulsion with the Tdia (E_Tdia), $137^a \pm 7$ nm for the emulsion with SDS (E_SDS) and $138^a \pm 8$ nm for the emulsion with Brij35 (Table 2). Accordingly, the droplet sizes were very similar, with no statistically significant differences observed regardless of the emulsifier. All emulsions exhibited a narrow monomodal size distribution in both intensity and number, with a relatively low PDI value (< 0.11) (Table 2).

Droplet size was measured immediately following emulsification (T_0) and after the physical stability test (T_F) at 37°C for 17 h. Statistical analyses revealed no significant droplet size difference between T_0 and T_F for emulsions without emulsifier (NoSF), with the Tdia, and with SDS, indicating excellent physical stability. A slight decrease (−20 nm) was observed for emulsions stabilized with Brij35, though the change remained minimal. These results showed that all emulsions exhibited high physical stability, with minimal droplet size variation during 17 h at 37°C. Thus, 10-h degradation kinetics of RA in the presence of different emulsifiers and in the absence of an emulsifier were performed (Figure 3).

The zeta potential of the emulsion with the cationic Tdia was $+48^b \pm 7$ mV, while the emulsion with SDS and Brij35 exhibited negative zeta potentials of $-44^a \pm 5$ mV and $-48^a \pm 3$ mV, respectively. The emulsion without an emulsifier showed a negative zeta potential of $-52^a \pm 3$ mV. The strong physical stability of emulsions was confirmed by their zeta potential values exceeding ±30 mV (Kulkarni et al. 2019). As expected, the cationic emulsifier (Tdia) resulted in an emulsion with a positive interface charge, confirming its ability to repel Fe^{2+}. Conversely, the anionic emulsifier (SDS) produced an emulsion

with a negative interface charge that can attract Fe^{2+}. However, the nonionic surfactant Brij35, which was expected to yield a very low zeta potential due to the absence of ionic groups (Boon 2014), exhibited a surprisingly high negative value. Additionally, the emulsion without any emulsifier followed the same trend, displaying a high negative zeta potential.

This results confirm RA's ability to self-organize in water without the need for an emulsifier and aligns with Bryl et al. (1998) and Drabent et al. (1997) work. Moreover, the negative charge of the droplets may stem from vitamin A impurities rather than the emulsifier itself, similarly to the finding of Lee and Choi (2021) who reported that the negative charge in fish oil emulsions at pH 7 was due to residual free fatty acids rather than the emulsifier.

Oxidative kinetics in the absence of iron (Figure 3a) showed that the degradation of RA in emulsions with different emulsifiers remained very similar up to 3 h. Beyond this point, slight but noticeable differences were observed. After 10 h, the retention of RA was $24^{a} \pm 3\%$ in the emulsion without emulsifier and $23^{a} \pm 1\%$ for SDS, showing that the use of this emulsifier had no impact on vitamin A oxidative stability. In contrast, for Brij35 and Tdia, retention values were respectively $30^{b} \pm 2\%$ and $37^{c} \pm 1\%$, showing that both Brij35 and Tdia improved RA stability. Since the emulsions had the same RA volume content, emulsifier concentration, and similar droplet sizes, the emulsifier concentration per unit of RA droplets should be similar. However, the coverage and thickness of the outer interfacial membrane can vary. This variation could be due to the specific volume of the hydrophilic head of each emulsifier. The cationic nature of Tdia's small polar heads is likely the primary stability factor. It stabilizes the emulsion by repelling positively charged pro-oxidant molecules. On the other hand, the large hydrophilic head of Brij35, which contains 23 ethylene oxide units, acts as a physical barrier slightly reducing oxidation.

These results align with most of the literature. Lycopene-based emulsions stabilized by anionic surfactants were shown to be more susceptible to oxidation due to their highly negative interface charge, followed by nonionic surfactants, while cationic surfactant-stabilized emulsions exhibited the slowest oxidation (Boon 2014). Similarly, oxidation rates were also found to be highest in salmon oil-in-water emulsions stabilized by anionic SDS, followed by nonionic Tween 20 and cationic dodecyltrimethylammonium bromide (DTAB) (Mancuso et al. 1999). Comparable results were obtained for corn oil-in-water emulsions, where SDS was shown to increase the oxidation rate compared to nonionic emulsifier (Mei et al. 1998). Additionally, after 10 h, in the presence of iron (Figure 3b), the retention of RA was $14.5^{d} \pm 0.4\%$ for the emulsion without emulsifier, $19.6^{a} \pm 0.4\%$ for SDS, $20^{a} \pm 1\%$ for Brij35, and $31^{b} \pm 3\%$ for the emulsion with the Tdia. The presence of iron slightly increased the degradation rate at the start of the kinetics for emulsions without antioxidant, with SDS, and with Brij35, which all have negative zeta potential values. This effect can be attributed to the attraction of Fe^{2+} ions to the negatively charged interface, where they act as prooxidants and catalyze hydroperoxide degradation. However, this increase in oxidation rate remained limited, confirming the hypothesis that only a few hydroperoxides were produced, hydroperoxides being primary oxidation products formed through a radical pathway. This aligns with Boon et al. (2010), who identified nine mechanisms of carotenoid oxidation and initial products. Among these nine pathways, only two led to peroxide radicals. In contrast, the kinetics of the emulsion stabilized with Tdia were less affected, as the cationic emulsifier's positive charge repels Fe^{2+} ions, limiting their prooxidant effect. Finally, the slight increase in stability of the emulsion with SDS and Brij35 compared to the one without emulsifier can be attributed to steric stabilization effects by forming a protective layer around the dispersed droplets, limiting the access of prooxidant species.

In our study, the limited effect of iron can be explained by the fact that, similar to carotenoids (Boon et al. 2010), vitamin A degradation may not primarily occur through the formation of hydroperoxides, which are typically converted by Fe^{2+} or Fe^{3+} into highly radical reactive species. This effect is even further reduced when using a cationic emulsifier. Thus, as with oils and carotenoids, the stability of vitamin A in emulsions may be slightly preserved by using cationic emulsifiers as iron prooxidant effect is inhibited. Despite the use of a cationic emulsifier, RA stability in these emulsions remains low, as over 60% of the compound is degraded by the end of the 10-h kinetic study (Figure 3). Consequently, the addition of AOx in emulsions stabilized with cationic emulsifiers was evaluated. The effect of iron addition is also assessed, as it may interact with AOx and potentially reduce their effectiveness.

3.3 | Effect of Antioxidant Addition

Vitamin A has been demonstrated to be highly susceptible to oxidation through exposure to air, water, heat, light, and acidic pH (Loveday and Singh 2008; Semenzato et al. 1994; Temova Rakuša et al. 2021). In an approach to identifying suitable AOx, we focused on two compounds that are well-established AOx with proven efficacy and industrial relevance, namely BHT and TOH. These AOx are well known for their strong ability to scavenge free radicals, protecting lipids and other sensitive compounds from oxidation. Their effectiveness has already been demonstrated for vitamin A, carotenoids, and oils (Banasaz et al. 2021; Boon 2014; Carlotti et al. 2002; Villeneuve et al. 2023). Additionally, CA, a phenolic compound found in rosemary extract, was selected. To the best of our knowledge, there is no information regarding the antioxidant efficiency of CA on vitamin A. However, it has been demonstrated to be an effective quencher of reactive oxygen species in lipid oxidation (Loussouarn et al. 2017) and to reduce low-density lipoprotein oxidation when combined with lycopene, though its efficiency was lower when used separately (Fuhrman et al. 2000).

The effects of antioxidant type and concentration were investigated in emulsions containing RA and Tdia surfactant in water, and compared with an emulsion without added antioxidant (noAox). The effect of iron addition was also assessed, as it may interact with AOx and potentially reduce their effectiveness. As the selected AOx are lipophilic, they were directly incorporated into the oil phase with RA in dioxane to maximize contact with the oxidizable substrate.

Physicochemical properties of the emulsions were characterized to ensure valid comparison of the AOx efficiency within this system (Table 2).

The z-average of emulsion without antioxidant (E_noAox) was $152^{abc} \pm 10$ nm (Table 2). The z-average of emulsions with BHT ranged between 153^{abc} nm (E_BHT2.5) and 173^{a} nm (E_BHT5) and from 155^{ab} nm (E_TOH2.5) to 171^{a} nm (E_TOH0.05) with TOH. The z-average of emulsions with CA ranged between 120^{d} nm (E_CA1) and 133^{bcd} nm (E_0.25). An increase in antioxidant concentration did not significantly change droplet size. Emulsions with BHT, TOH, and noAox resulted in similar droplet sizes, as their z-average means by antioxidant were not significantly different, falling within statistical group α (Table 2). However, the z-average means of emulsions with CA were significantly different compared to all others, as they fell within a second statistical group β, indicating smaller droplet sizes.

All emulsions with and without AOx exhibited narrow monomodal size distribution in both intensity and number with a PDI of less than 0.1 (Table 2). Despite minor differences in droplet size, all emulsions exhibited similar physicochemical properties, allowing for a valid comparison of oxidative kinetics. This study particularly focuses on the initial 50% of the degradation kinetics, as this phase is often considered critical for assessing the oxidative stability of applied products and their shelf life.

The oxidative kinetics of RA emulsions depending on antioxidant nature and concentration showed that the presence of an antioxidant generally increased stability with increasing concentrations (Figure 4). Only the emulsion with 0.25 μM of CA (E_CA0.25) appeared to slightly enhance oxidation, and this effect was even more pronounced in the presence of iron (E_CA0.25_Fe). This phenomenon could be linked to the pro-oxidant behavior of CA under certain conditions (Frankel et al. 1996). Indeed, while CA is widely recognized for its antioxidant properties, it has also been shown to increase DNA damage in the bleomycin assay, indicating a pro-oxidant effect (Aruoma et al. 1992) and to generate radical intermediates in the presence of oxidized lipids (Geoffroy et al. 1994). Additionally, at TOH concentration 5 μM, stability in the presence of iron declined rapidly toward the end of the kinetics. This phenomenon could be attributed to the accelerated formation of oxidized TOH derivatives, which are highly reactive (Barouh et al. 2022).

Moreover, in all emulsions, RA began degrading immediately at the start of the kinetics, with no observable lag phase. This suggests that the oxidation process is not effectively delayed, even in the presence of powerful radical scavenging AOx. While none of the tested AOx completely prevented RA degradation, their effect became noticeable later in the kinetics, where they reduced the oxidation rate rather than fully inhibiting it. The impact of iron remained very limited and was attributed to the cationic Tdia charge.

Directly comparing the degradation kinetics of RA between the different AOx in Figure 4 can be challenging. To facilitate this comparison, we extracted two key parameters from the kinetic curves: the time at which 25% degradation is reached ($T_{25\%}$) and the retention percentage after a 10-h oxidation period (R_{10h}). This approach provides a more quantitative way to assess antioxidant efficiency by capturing both the initial resistance to oxidation and the overall stability over time, where higher $T_{25\%}$ and R_{10h} values indicate greater protection against degradation. The corresponding values are presented in Figures 5 and 6.

In the absence of iron, the time required to reach 25% oxidation ($T_{25\%}$) for emulsions without any antioxidant was $2.32^{a} \pm 0.29$ h (Figure 5a). $T_{25\%}$ was the highest for TOH with $8.39^{b} \pm 0.38$ h at 5 μM. Comparatively, the highest $T_{25\%}$ obtained with BHT and CA was $3.30^{c} \pm 0.46$ h and $3.61^{c} \pm 0.26$ h, respectively, also at 5 μM. During the initial phase of oxidation, TOH, BHT, and CA at 5 μM all improved RA retention, as the time to reach 25% degradation significantly increased compared to emulsions without AOx (Figure 5). While the effect is clearly marked for TOH, it remained very limited for BHT and CA, highlighting the limited effectiveness of these AOx in the early stage of degradation.

In the presence of iron, the $T_{25\%}$ for emulsions without any antioxidant was $1.57^{a} \pm 0.15$ h (Figure 5b). The highest $T_{25\%}$ was again observed for TOH, reaching $8.55^{b} \pm 1.31$ h at 5 μM. Comparatively, the highest $T_{25\%}$ values for BHT and CA were $2.94^{c} \pm 0.31$ h and $2.67^{ac} \pm 0.13$ h respectively, also at 5 μM. TOH exhibited the greatest ability to delay the initial phase of oxidation, while the effect of BHT remained low and was nonsignificant for CA. $T_{25\%}$ remained statistically unchanged compared to iron-free values. Iron did not impair the effectiveness of the AOx. The minimal impact of iron in this system could once again be attributed to the presence of cationic Tdia emulsifier.

The limited effectiveness of free radical scavenging AOx refines our hypothesis on the initial degradation pathways of RA. Since none of the three tested AOx could fully prevent early degradation, this suggests that vitamin A degradation initially occurs through a mechanism that is not predominantly radical-driven. This hypothesis is consistent with the previous results, where few hydroperoxides would be formed, as hydroperoxides mainly involve radical-driven mechanisms. Moreover, the higher efficiency of TOH, the most apolar antioxidant, to prevent early oxidation could be attributed to its close localization with RA facilitating the deactivation of singlet oxygen into triplet oxygen after energy or charge transfer (Barouh et al. 2022). Indeed, the solvent-displacement emulsification method does not allow for precise control over the localization of AOx within the emulsion. Based on their relative lipophilicity to RA, it can be expected that TOH (Log $p = 9.2$) remains primarily within RA droplets (Log $p = 8.8$–9.4), while BHT (Log $p = 5.1$) and CA (Log $p = 4.5$–5.1), being less lipophilic, preferentially localize at the oil–water interface. Conversely, the more limited effect of BHT and CA may result from their suboptimal positioning in the emulsion.

The remaining RA percentage after a 10-h oxidation period (R_{10h}) in the absence of iron for emulsions without any antioxidant was $45^{a} \pm 3\%$ (Figure 6a). R_{10h} was the highest for TOH, with a retention of $73^{b} \pm 2\%$ at 5 μM. Comparatively, the highest R_{10h} obtained with BHT and CA was $61^{c} \pm 1\%$ and $65^{c} \pm 1\%$, respectively at 5 μM. After a 10-h kinetics, TOH, BHT, and CA all significantly enhanced the retention of RA. TOH allowed for the highest retention, closely followed by CA and BHT.

In presence of iron, the remaining RA percentage after 10 h (R_{10h}) for emulsions without any antioxidant was $39^{\alpha} \pm 4\%$ (Figure 6b). R_{10h} was the highest for TOH with a retention of $70^{b} \pm 1\%$ at 2.5 μM. Comparatively, the highest R_{10h} obtained with BHT and CA was $60^{c} \pm 1\%$ and $57^{c} \pm 1\%$, respectively, at 5 μM. The minimal impact of iron in this system could once again be attributed to the presence of cationic Tdia emulsifier.

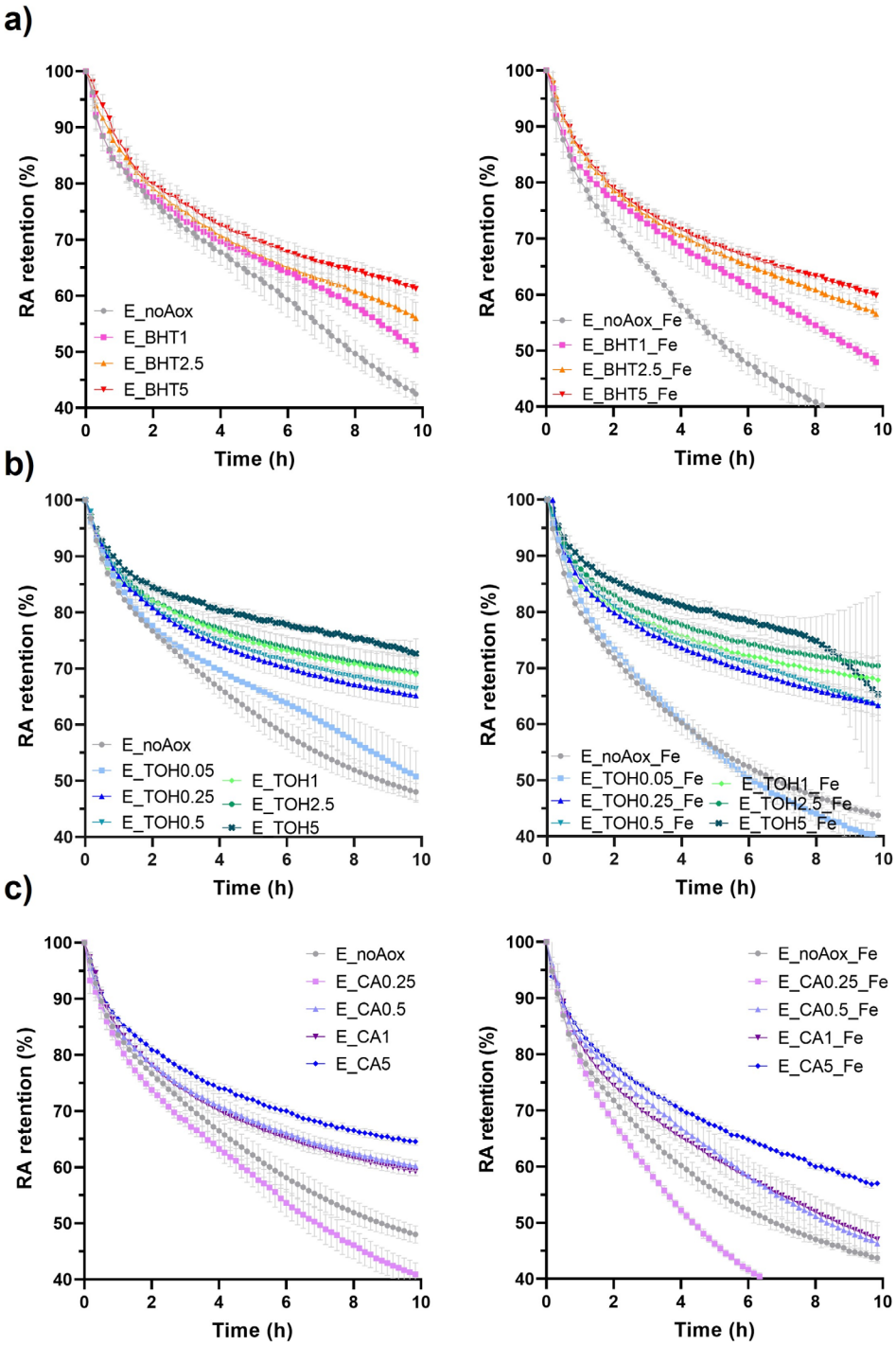

FIGURE 4 | Oxidative kinetics of retinyl acetate emulsions (a) with BHT, (b) α-tocopherol, (c) carnosic acid, in the absence (left) and in the presence (right) of iron (_Fe) (for used abbreviations see Table 1).

These results suggest that part of the oxidation was successfully inhibited by the free radical scavenger, confirming the involvement of the radical oxidation pathway. The effectiveness of TOH in protecting vitamin A from oxidation in O/W emulsions was similarly demonstrated (Banasaz et al. 2021), while the protective role of BHT against vitamin A in O/W emulsions was also reported (Carlotti et al. 2002). The effectiveness of CA was found to be comparable to that of BHT, making it a promising natural alternative. However, caution must be taken with its use as it was pro-oxidant at low concentrations. In this study, its potential may even be underestimated due to the smaller emulsion droplet sizes obtained (Table 2). Indeed, smaller droplet sizes lead to a higher surface area, which can accelerate oxidation rates.

Nevertheless, for all three AOx, the difference between maximum and minimum retention (reference without antioxidant) remained relatively small, further highlighting their limited effectiveness and suggesting the involvement of multiple oxidation pathways. This includes the predominance of a non-radical oxidation pathway in the early degradation phase,

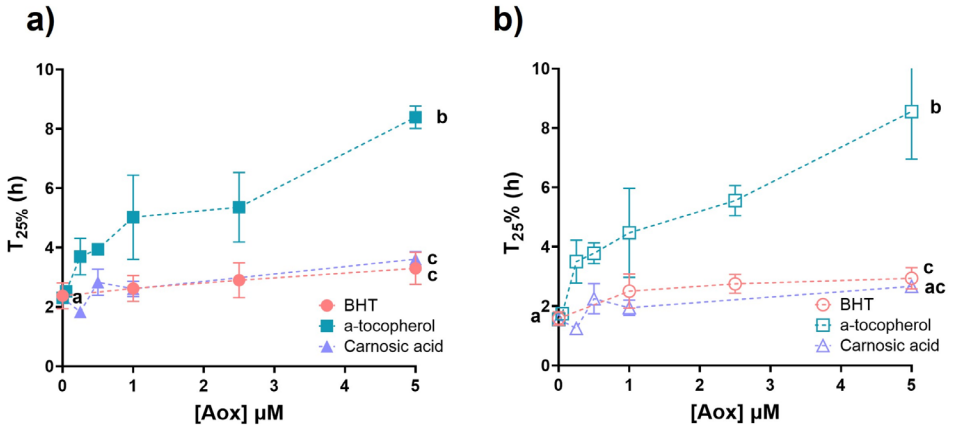

FIGURE 5 | Specific time when 25% of retinyl acetate oxidation is reached ($T_{25\%}$) (a) in absence of iron, (b) in the presence of iron.

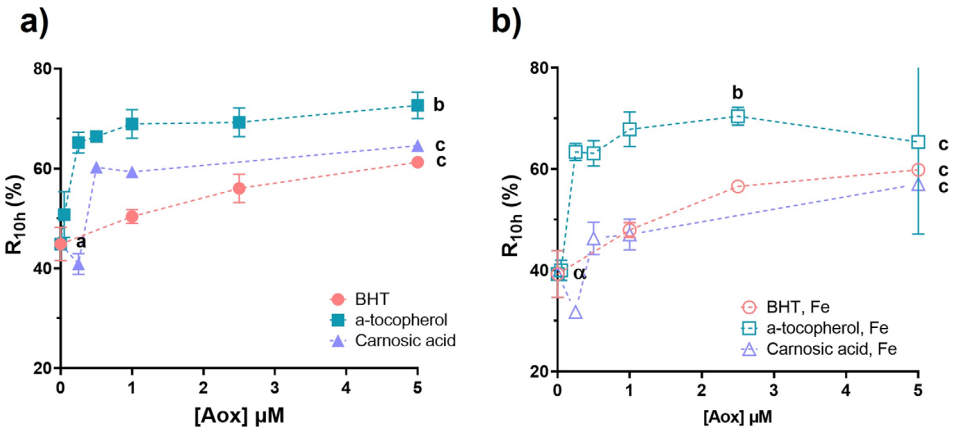

FIGURE 6 | Retention of retinyl acetate after 10 h of kinetics (R_{10h}) (a) in the absence of iron, (b) in the presence of iron.

followed by radical-driven pathways. This aligns with Xu and Watson (2021), who found that adding AOx such as BHT, BHA, ascorbic acid, and tocopherols had little or no effect on vitamin A stability under natural oxidation. In addition, this hypothesis aligns with Paquette and Kanaan (1985) results, who observed spectral changes during the reaction of RA in a simple solvent system, indicating the involvement of multiple degradation mechanisms. Finally, this result aligns with the known oxidation pathways of carotenoids, which occur through both radical-and nonradical-driven mechanisms (Boon et al. 2010).

4 | Conclusion

This study provides new insights into the oxidative stability of vitamin A in emulsified systems in the selected experimental conditions (temperature, concentration in vitamin A, type of emulsifier). Among the tested vitamin A forms, RP exhibited the highest stability, followed by RA and RO, likely due to structural and electronic factors. The use of cationic emulsifiers demonstrated efficacy in repelling pro-oxidant metal ions, thereby reducing vitamin A oxidation. Additionally, TOH, BHT, and CA were found to enhance vitamin A stability, with TOH demonstrating the greatest effectiveness. However, the limited efficiency of these AOx facilitated the identification of two distinct oxidation pathways: the predominance of a nonradical oxidation pathway in the early degradation phase, followed by radical-driven pathways. The weak catalytic effect of iron, attributed to electrostatic repulsion in cationic-stabilized emulsions and low hydroperoxide production, further supports the hypothesis of a nonradical degradation mechanism in early oxidation stages.

These findings refine our understanding of vitamin A oxidation and highlight key formulation strategies for its stabilization. Future research should focus on AOx targeting nonradical oxidative pathways and further elucidating the interplay between formulation factors and oxidation mechanisms to develop more effective stabilization approaches.

Author Contributions

M. De Vreese: conceptualization, methodology, investigation, formal analysis, writing – original draft. **E. Durand:** conceptualization, investigation, writing – original draft. **B. Baréa:** methodology, investigation, formal analysis, review editing. **D. Morvan:** conceptualization, resources, review editing. **C. Aleman:** conceptualization, resources, methodology, review editing. **J. Lecomte:** conceptualization, supervision, investigation, writing – review and editing. **P. Villeneuve:** conceptualization, supervision, investigation, writing – review and editing. All authors contributed to and approved the final draft of the manuscript.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

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